Thrombosis Research 177 (2019) 70–78

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Full Length Article

Aberrant expression of microRNA in CD4$^+$ cells contributes to Th17/Treg imbalance in primary immune thrombocytopenia☆

Mingqiang Hua[a,1], Ju Li[a,1], Chunyan Wang[a,b], Linlin Shao[a], Ming Hou[a,c,d], Jun Peng[a,c], Qi Feng[a,*]

[a] Department of Hematology, Qilu Hospital, Shandong University, China
[b] Department of Emergency, Second Hospital of Shandong University, Jinan, China
[c] Shandong Provincial Key Laboratory of Immunohematology, Qilu Hospital, Shandong University, Jinan, China
[d] Leading Research Group of Scientific Innovation, Department of Science and Technology of Shandong Province, Qilu Hospital, Shandong University, Jinan, China

ARTICLE INFO

Keywords:
Immune thrombocytopenia
miRNA
Treg cell
Th17 cell

ABSTRACT

Introduction: Imbalance of T helper 17 (Th17) cells and regulatory T (Treg) cells occurs in primary immune thrombocytopenia (ITP), but the mechanism remains unclear. We investigated whether expression of microRNAs (miRNAs) related to helper T or Treg cells regulate the Th17/Treg ratio in CD4$^+$ T cells.

Materials and methods: Peripheral blood was obtained from 52 active ITP patients and 56 healthy controls. We detected miRNA expression using RT-PCR with stem-loop primers and U6 as control.Th17 and Treg percentages were analyzed by flow cytometry. CD4$^+$ cells were transfected with miRNA (miR-99a, miR-182-5p, miR-183-5p) mimics or inhibitors to investigate their function.

Results: miR-99a expression in CD4$^+$ cells in ITP patients was lower than in controls, while expression of miR-182-5p and miR-183-5p were higher in ITP patients. Moreover, Treg percentage correlated positively with miR-99a expression in ITP patients. We found no significant correlation between Th17 percentage and miR-182-5p or miR-183-5p expression. miR-183-5p expression correlated negatively with platelet count, while we found no significant difference between platelet count and miR-99a or miR-182-5p. miR-183-5p expression in CD4$^+$ T cells from severe patients was significantly higher than in those from non-severe patients. Furthermore, down-regulating miR-183-5p expression repressed Th17 differentiation, while up-regulating miR-99a increased Tregs detected in CD4$^+$ cells from ITP patients. In addition, up-regulated miR-99a repressed mTOR and p-mTOR expression.

Conclusions: miR-99a, miR-182-5p, and miR-183-5p expression levels in CD4$^+$ cells were abnormal in ITP patients. Aberrant expression of miRNAs may contribute to the Th17/Treg imbalance in ITP patients and may represent a novel therapeutic target.

1. Introduction

Primary immune thrombocytopenia (ITP) is an acquired autoimmune disease characterized by a reduced platelet count and an increased risk of bleeding in the absence of an obvious initiating and/or underlying cause [1]. The main mechanisms include autoantibody-based platelet destruction and decreased platelet production.

Recently, T cell imbalance has been recognized as an important mechanism of ITP. Studies identified an elevated Th1/Th2 ratio and Th1 cytokine profiles in ITP patients [2]. T helper 17 (Th17) cells have been identified as a distinct subset of effector helper T cells that play a pro-inflammatory role during autoimmune diseases as well as host defense against extracellular pathogens, in contrast, regulatory T (Treg) cells have an antagonist effect. More recently, evidence has suggested that Th17 and Treg cells are important T subsets that are responsible for ITP pathogenesis. Increased Th17 cells and cytokine IL-17 have been found in ITP patients [3,4]. In addition, an impaired number and activity of Treg cells were found in ITP patients [5,6]. Th17/Treg balance influences the outcome of immune responses and provides a basis of understanding in the mechanism of autoimmune and autoinflammatory diseases [7,8]. Although growing studies have found Th17/Treg imbalance in plenty of autoimmune diseases, including ITP, the molecular mechanism has not been well defined.

MicroRNAs (miRNAs) belong to a class of small noncoding RNAs

☆ Abbreviations: ITP, Th17, Treg, miRNA, PBMC.

* Corresponding author at: Department of Hematology, Qilu Hospital, Shandong University, 107 West Wenhua Rd, Jinan 250012, China.
E-mail address: fengqi0313@163.com (Q. Feng).

[1] These two authors contributed equally to this work.

https://doi.org/10.1016/j.thromres.2019.03.005
Received 7 December 2018; Received in revised form 23 February 2019; Accepted 4 March 2019
Available online 05 March 2019
0049-3848/

with only approximately 21 nucleotides. miRNAs negatively modulate gene expression at the posttranscriptional level. In their mature form, miRNAs pair to sequences in the 3′-untranslated region (UTR) of mRNAs, leading to translational repression or mRNA degradation. miRNAs participate in regulation of autoimmunity, including activation, function, and homeostasis of effector T cells or Treg cells [9,10]. Accumulating evidence shows that miRNAs are associated with development and function of Th17 and Treg cells. For example, miR-183-96-182 cluster is highly expressed in Th17 cells, while pathogenic cytokine production from Th17 cells enhances during autoimmunity [11,12]. In addition, incubating Treg cells with IL-6 leads to increased miR-17 expression and diminished suppressive activity [13]. miR-17 can also exert critical dual roles, promoting Th1 responses and preventing inducible Treg differentiation [14]. Recent study revealed that miR-99 promotes Treg cell differentiation cooperatively with miR-150 [15]. Another recent study revealed that miR-146 negatively regulates TLR/IL-1R signaling pathway [10]. It has been shown that IL-1R signaling pathway facilitates Th17 differentiation and IL-17 production [16]. When it comes to miR-155, recent studies have identified that miR-155 contributes to Th17 cells differentiation in colitis mice [17] and autoimmune encephalomyelitis mice [18], while Heyn et al. revealed that miR-155 enhances differentiation of Treg cells in patients with neuropathic pain [19]. Besides, Ghorbani et al. revealed that miR-181 promotes Treg differentiation in the central nervous system of patients with multiple sclerosis [20]. Moreover, miR-326 promotes Th17 differentiation and is highly associated with disease severity in patients with multiple sclerosis [21]. Therefore, we retrieved several miRNAs from databases (miRBase, starBase, Tarbase) to explore the mechanism of the Th17/Treg imbalance, including miR-17-5p, miR-99a, miR-146-5p, miR-155-5p, miR-181-5p, miR-183-96-182 cluster, and miR-326 [11,18,21,22].

We are eager to understand the function of miRNAs in Th17 and Treg cell differentiation and expansion in ITP, as they may contribute to the imbalance of T cell subsets. We hypothesize that miRNAs take part in creating the Th17/Treg imbalance in ITP. Here, we sought to investigate whether Th17 or Treg cell-related miRNAs regulate the ratio of Th17/Treg in $CD4^+$ T cells, as well as how miRNAs regulate the Th17/Treg balance. Furthermore, we examined the association between the miRNAs and clinical implications of ITP patients.

2. Methods

2.1. Patients and controls

Fifty-two active ITP inpatients (28 females and 24 males; age 18–66 years, median 34 years) were recruited between June 2016 and January 2019 from the Department of Hematology, Qilu Hospital, Shandong University, Jinan, China. ITP patients were diagnosed by history, clinical manifestations, physical examinations, radiologic findings and laboratory findings according to the international consensus [23]. Patients enrolled in our study were all chronic ITP patients who had been diagnosed with ITP for > 12 months. Active ITP patients refer to chronic patients hospitalized for the presence of bleeding symptoms or the platelet count $< 30 \times 10^9$/L in active stage of ITP. Peripheral blood was collected from patients who were admitted into hospital without receiving any ITP-specific treatments (corticosteroids, IVIG, Rituximab, and TPO-RA, etc.) within 3 months. In addition, patients were stratified by severity and corticosteroid sensitivity according to the standardization of terminology, definitions, and outcome criteria [24]. Patients who have clinical relevant bleeding at presentation sufficient to mandate treatment, or show new bleeding symptoms requiring additional therapeutic intervention with a different platelet-enhancing agent or an increased dose are classified as having severe ITP. Corticosteroid sensitivity is defined as a platelet count $\geq 30 \times 10^9$/L with at least a 2-fold increase from the baseline count and without bleeding after corticosteroid management. Requirements for additional interventions were considered as corticosteroid resistance. For the control group, 56 healthy participants were enrolled in our study (29 females and 27 males; age 20–62 years, median 36 years; Table 1).

Table 1
Clinical characteristics of ITP patients and controls.

Parameters	ITP	Controls
	(n = 52)	(n = 56)
Gender (male: female)	24:28	27:29
Age (years, median, range)	34(18–66)	36(20–62)
Blood routine		
Platelet(1×10^9/L; median, range)	16(1 − 30)	248 (155–425)
WBC(1×10^9/L; median, range)	6.25(4.1–10.1)	6.8 (4.2–9.9)
HGB(g/L; median, range)	78(65–135)	141 (123–168)
Stage		
Chronic ITP	52(100%)	
Severity		
Severe ITP	29	
Non-severe ITP	23	
Response to dexamethasone		
Sensitive	25	
Resistant	19	

Cases complicated by diabetes, hypertension, cardiovascular diseases, pregnancy, active infection, connective tissue diseases or other autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, were excluded from the study. The study was performed with the approval of the Medical Ethical Committee of Qilu Hospital, Shandong University. Written informed consent was obtained from each participant in accordance with the Declaration of Helsinki.

2.2. Preparation of plasma and $CD4^+$ cells

Peripheral blood samples were collected into tubes containing ethylenediaminetetraacetic acid (EDTA). Plasma was isolated from whole blood by centrifugation (350 ×*g*, 5 min), then subjected to high speed centrifugation (12,000 ×*g*, 2 min) and finally stored at −80 °C for the measurement of cytokines. Peripheral blood mononuclear cells (PBMCs) were separated from blood by gradient centrifugation (750 ×*g*, 20 min) on Ficoll-Paque (Pharmacia Diagnostics). Cells were washed twice and then $CD4^+$ T cells were separated with anti-CD4 MACS (magnetic-activated cell sorting) microbeads and MACS columns (Miltenyi Biotec) according to the manufacturer's instructions. The purity of $CD4^+$ cells was > 95% according to flow cytometry. Cells were subsequently used for RNA isolation, flow cytometry, or miRNAs transfection.

2.3. RNA isolation, reverse transcription, and quantitative RT-PCR

Isolation of total RNA from $CD4^+$ T cells was performed using TRIzol (Invitrogen). RNA concentration and purification were assessed using a spectrophotometer (Eppendorf, GER).

To detect miRNA expression, reverse transcription was performed using the PrimeScript™ RT reagent Kit with gDNA Eraser (Code No. RR047Q, TaKaRa, Japan) according to manufacturer's instructions. The reverse transcription reaction includes 42 °C for 2 min to eliminate the genomic DNA, then 37 °C for 15 min, and 85 °C for 5 s.

Quantitative RT-PCR (reverse transcription polymerase chain reaction) was performed for miRNAs and small nuclear RNA U6 on a Roche LightCycler 480II instrument and TaqMan Universal Master Mix (Applied Biosystems). After pre-denaturation (95 °C, 5 min), PCR reactions were cycled 40 times through denaturation (95 °C, 20 s) and extension (62 °C, 40 s). Primers and probes were all provided from TaqMan miRNA Assays. Primers are detailed in Table 2. The amplification efficiency between the target and the reference control were compared using the ΔCt calculation.

Table 2
Gene primers in the study.

Gene name	Forward primer	Reverse primer
U6	CAGCACATATACTAAAATTGGAACG	ACGAATTTGCGTGTCATCC
hsa-mir-17-5p	ATTCTTCCAAAGTGCTTACAGTGC	TATGGTTTTGACGACTGTGTGAT
hsa-mir-183-5p	CGAACGATATGGCACTGGTAGA	TATGGTTTTGACGACTGTGTGAT
hsa-mir-96-5p	CGAACTTTGGCACTAGCACATT	TATGGTTTTGACGACTGTGTGAT
hsa-mir-182-5p	ATCACTTTTGGCAATGGTAGAACT	TATGGTTTTGACGACTGTGTGAT
hsa-mir-99a	CATTACTAAACCCGTAGATCCGAT	TATGGTTTTGACGACTGTGTGAT
hsa-mir-146a-5p	TAATCGTGTGAGAACTGAATTCCA	TATGGTTTTGACGACTGTGTGAT
hsa-mir-155-5p	ACGCTCAGTTAATGCTAATCGTGATA	TATGGTTTTGACGACTGTGTGAT
hsa-mir-181a-5p	CACTCTCTCTCAGAAACATTCAAC	TATGGTTTTGACGACTGTGTGAT
hsa-mir-326	AATCTTACCTCTGGGCCCTTC	TATGGTTTTGACGACTGTGTGAT

2.4. Percentage of Th17 and Treg analysis by flow cytometry

PBMCs from ITP patients and healthy controls were incubated for 4 h in RPMI-1640 medium containing 10% fetal bovine serum (Gibco, Australia), phorbol myristate acetate (50 ng/ml), ionomycin (1 μg/ml) and Brefeldin A (4 μg/ml; all from Alexis Biochemicals, San Diego, CA, USA). To identify Th17 cells, cells were stained with PE-Cy5-conjugated anti-CD3 monoclonal antibody (mAbs) and eFluor780-conjugated anti-CD8 mAbs (room temperature, 30 min, dark). After fixation and permeabilization, cells were intracellularly stained with FITC-conjugated anti-IFN-γ mAbs and PE-conjugated anti-IL-17A mAbs (4 °C, 40 min, dark). To stain Treg cells, cells were incubated with FITC-conjugated anti-CD4 and APC-conjugated anti-CD25 mAbs (room temperature, 30 min, dark). After fixation and permeabilization, cells were stained with PE-conjugated anti-FoxP3 mAbs (1 h). The same-species, same-isotype IgG was used as an isotype control. We gated $CD3^+CD8^-$ lymphocytes and analyzed the proportion of Th17 ($CD3^+CD8^-IFN\text{-}\gamma^-IL17A^+$) and Treg cells ($CD3^+CD8^-CD25^+FoxP3^+$) among $CD3^+CD8^-$ lymphocytes. All antibodies used for flow cytometry were purchased from eBioscience, Thermo Fisher Scientific, USA.

2.5. Cytokine quantification by ELISA

IL-10, IL-17, and TGF-β concentrations in plasma were tested using enzyme linked immunosorbent assay (ELISA) kits following the manufacturer's instructions (eBioscience, Thermo Fisher Scientific, USA). Two replicate wells were quantified per sample.

2.6. In vitro culture of $CD4^+$ T cells and transfection of miRNAs

Eight patients were randomly selected from chronic ITP patients who were admitted into hospital without receiving any treatment within 3 months. To investigate the function of miRNAs in the differentiation of $CD4^+$ cells, isolated $CD4^+$ T cells from the eight ITP patients were transfected with miR-99a mimics, miR-182-5p inhibitor, miR-183-5p inhibitor, or negative control (Shanghai Genepharma, China). We used EndoFectin-Max Kits (GeneCopoeia, USA) together with OPTI-MEM Reduced Serum Mediun (Gibco, USA) according to manufacturer's instructions. After 6 h, medium was changed to Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal bovine serum (Gibco, Australia). Quantitative RT-PCR was performed to verify up- or down-regulation of target miRNAs using TaqMan miRNA assays. Cells were cultured in plate-bound anti-human CD3 and soluble anti-human CD28(2 μg/ml) plus IL-2(50 U/ml; in humidified air, 5% CO_2, 37 °C). After 4 days, cells were harvested and analyzed for Th17 and Treg cell differentiation by flow cytometry.

2.7. Immunoblotting

After transfection of miR-99a for 72 h, $CD4^+$ T cells were PBS-washed and incubated for 15 min on ice with NP-40 lysis buffer (Beyotime Biotechnology, China). After sonication, lysate was cleared by centrifugation (15 min, 15,000 × *g*, 4 °C). Lysate protein content was determined using BCA protein quantification assay (Beyotime Biotechnology, China). For immunoblotting, 30 μg of total protein was loaded per lane and detection was performed according to standard protocols. Monoclonal antibodies directed against mTOR (#2983), p-mTOR (#5536) and β-actin (#4970) were purchased Cell Signaling Technology, USA.

2.8. Statistical analysis

Data are expressed as median or mean. Significant differences between groups were determined using Mann-Whitney Test or Student's *t*-tests. A two-tailed $p < 0.05$ was considered statistically significant. Data were analyzed using SPSS 21.0 software (SPSS Inc., Chicago, IL, USA). * represents $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$; ns: not significant.

3. Results

3.1. Aberrant miRNAs expression in ITP patients

Previous studies reported that miRNA expression is aberrant in ITP patients, especially in PBMCs [25–27]. However, expression of miRNAs in human $CD4^+$ lymphocytes has not been well studied. We performed comparative analysis of miRNAs levels of miR-17-5p, miR-99a, miR-96-5p, miR-146a-5p, miR-155-5p, miR-181a-5p, miR-182-5p, miR-183-5p, and miR-326 in active ITP patients and age-matched healthy controls (Table 3). Relative miR-99a expression was lower in $CD4^+$ cells from ITP patients than in those from controls (median 37.37 vs 66.67, $p < 0.001$; Fig. 1A). In addition, miR-182-5p and miR-183-5p expression levels were higher in cells from active ITP patients than in those from controls (miR-182-5p, median 16.2 vs 6.09, $p < 0.001$, Fig. 1B; miR-183-5p, median 5.59 vs 2.58, $p < 0.01$; Fig. 1C).

Among the miRNAs, we found a positive correlation between miR-182-5p and miR-183-5p expression levels ($p < 0.01$, $r = 0.468$), but found no significant correlation between miR-99a and miR-182-5p or

Table 3
Relative expression of miRNA in ITP patients and controls.

miRNA	ITP (n = 52)		Control (n = 56)		p value
	Median	Range	Median	Range	
miR-17-5p	13.7	2.08–97.1	14.84	1.21–78	ns
miR-96-5p	8.68	0.5–95.4	9.09	2.14–40.03	ns
miR-99a	37.37	7.65–161.6	66.67	5.53–297	< 0.001
miR-146-5p	5.01	2.38–17.12	4.664	0.06–40.1	ns
miR-155-5p	4.66	1.14–13.97	4.48	1.54–16.55	ns
miR-181-5p	1.92	0.84–6.88	2.22	0.41–9.82	ns
miR-182-5p	16.2	1.02–88.3	6.09	1.00–29.97	< 0.001
miR-183-5p	5.59	0.96–67.3	2.58	0.01–22.39	< 0.01
miR-326	3.29	0.4–35.2	4.12	1.09–11.8	ns

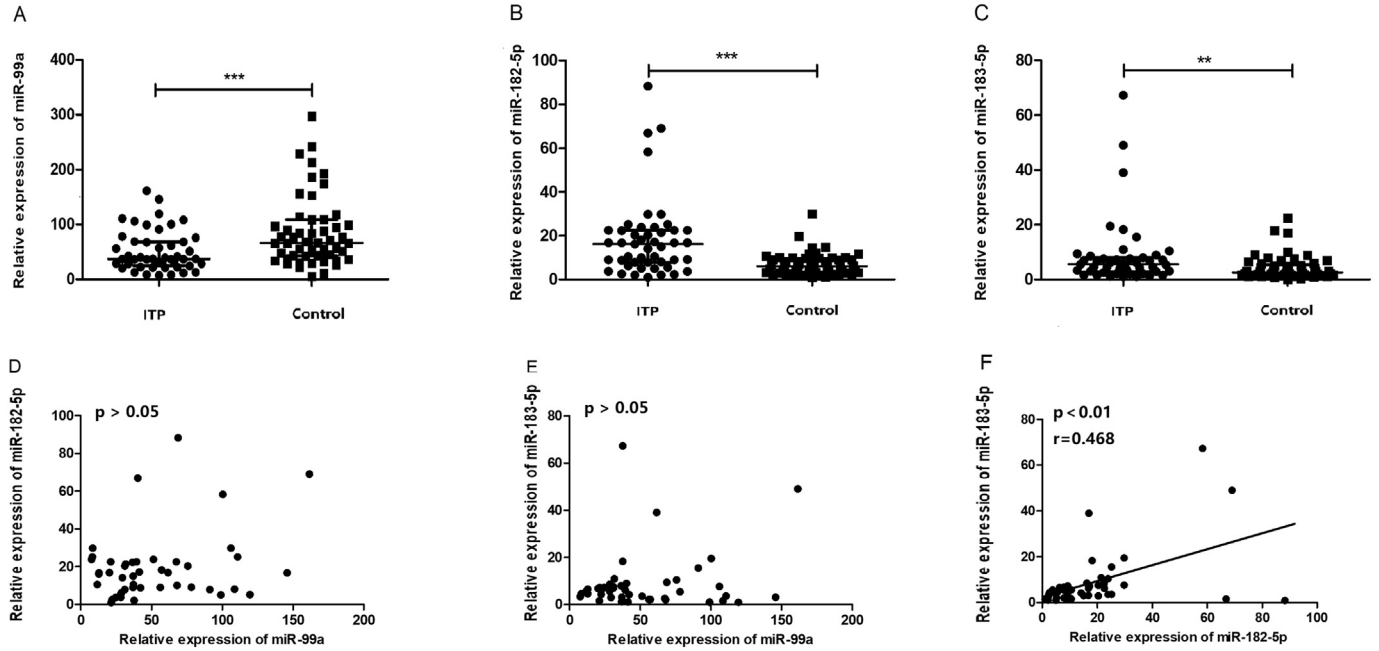

Fig. 1. Relative expression of miRNA expression in ITP patients and controls. (A–C) Relative expression of miRNAs, miR-99a, miR-182-5p, and miR-183-5p, in $CD4^+$ cells from ITP patients and healthy controls. (D–F) Correlation analysis among miRNAs. Median with interquartile range.

miR-183-5p ($p > 0.05$; Fig. 1D–F). In addition, the expression of other miRNAs including miR-17-5p, miR-96-5p, miR-146-5p, miR-155-5p, miR-181-5p, and miR-326 did not differ significantly compared to healthy controls ($p > 0.05$; Supplemental Fig. 1).

3.2. Th17/Treg imbalance in ITP patients

Consistent with previous findings, we found aberrant percentages of Th17 and Treg cells in ITP patients compared to healthy controls [28,29]. The percentage of Th17 cells was increased in ITP patients (median 2.89% vs 1.80%, $p < 0.01$; Fig. 2A–E), while the frequency of Treg cells was decreased in ITP patients compared to controls (median 1.96% vs 3.85%, $p < 0.01$; Fig. 2F–J).

miR-99a is involved in differentiation of Treg cells, while miR-182-5p and miR-183-5p regulate Th17 cells [11,15]. Thus, we hypothesized that these miRNAs may contribute to the imbalance of Treg and Th17 cells in ITP. We found a positive correlation between the percentage of Treg cells and miR-99a expression in ITP patients ($r = 0.445$, $p < 0.05$; Fig. 3A). However, there was no significant difference between Th17 and miR-182-5p or miR-183-5p in ITP patients (Fig. 3B, C). The Treg/Th17 ratio was decreased in active ITP patients compared to controls (median 0.72 vs. 1.68; $p < 0.001$; Fig. 3D). Interestingly, the decreased trend of miR-99a/miR-182-5p or miR-99a/miR-183-5p was in consistence with the Treg/Th17 ratio ($p < 0.001$; Fig. 3E, F).

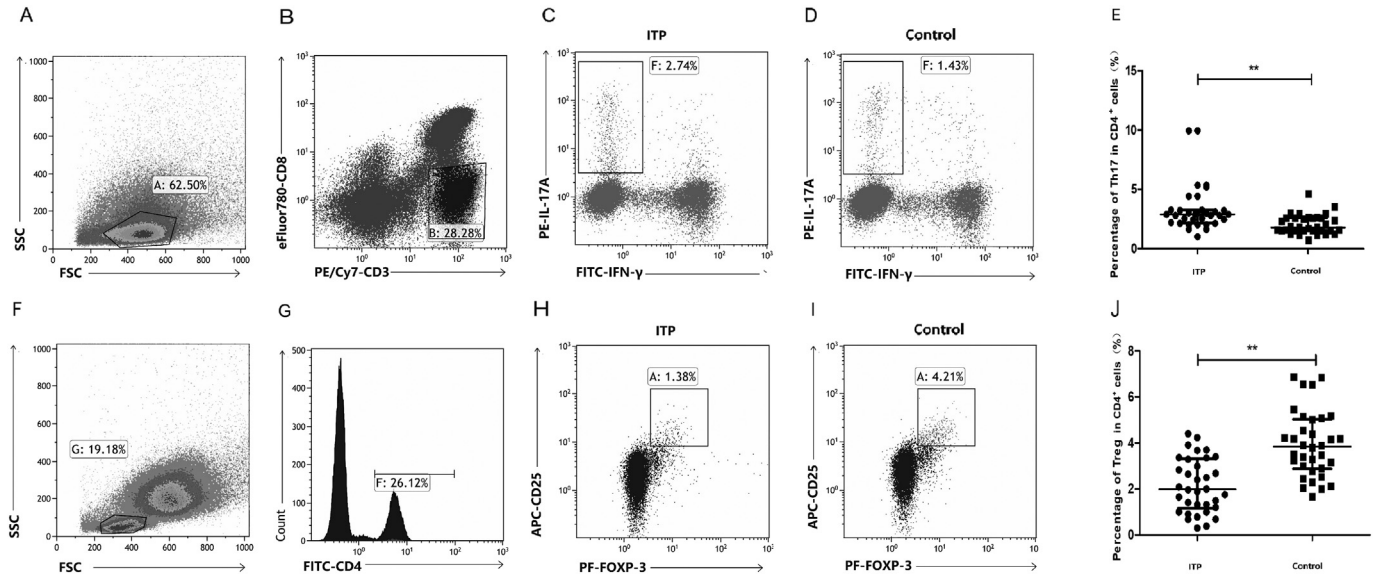

Fig. 2. Percentage of Th17 and Treg cells in ITP patients and healthy controls. (A and F) Lymphocytes. (B) Gate "B": $CD3^+CD8^-$ T lymphocytes represents $CD4^+$ T cells. (C and D) Gate "F": representative scattergrams of $IL\text{-}17^+IFN^-\gamma^-$ on $CD3^+CD8^-$ T cells in ITP patients and healthy controls, respectively. (E) Proportion of Th17 cells from ITP patients and healthy controls. (G) Gate "F": $CD4^+$ T lymphocytes. (H and I) Gate "A": representative scattergrams of $FOXP3^+CD25^+$ on $CD4^+$ cells in ITP patients and healthy controls, respectively. (J) Proportion of Treg cells from ITP patients and healthy controls. Median with interquartile range.

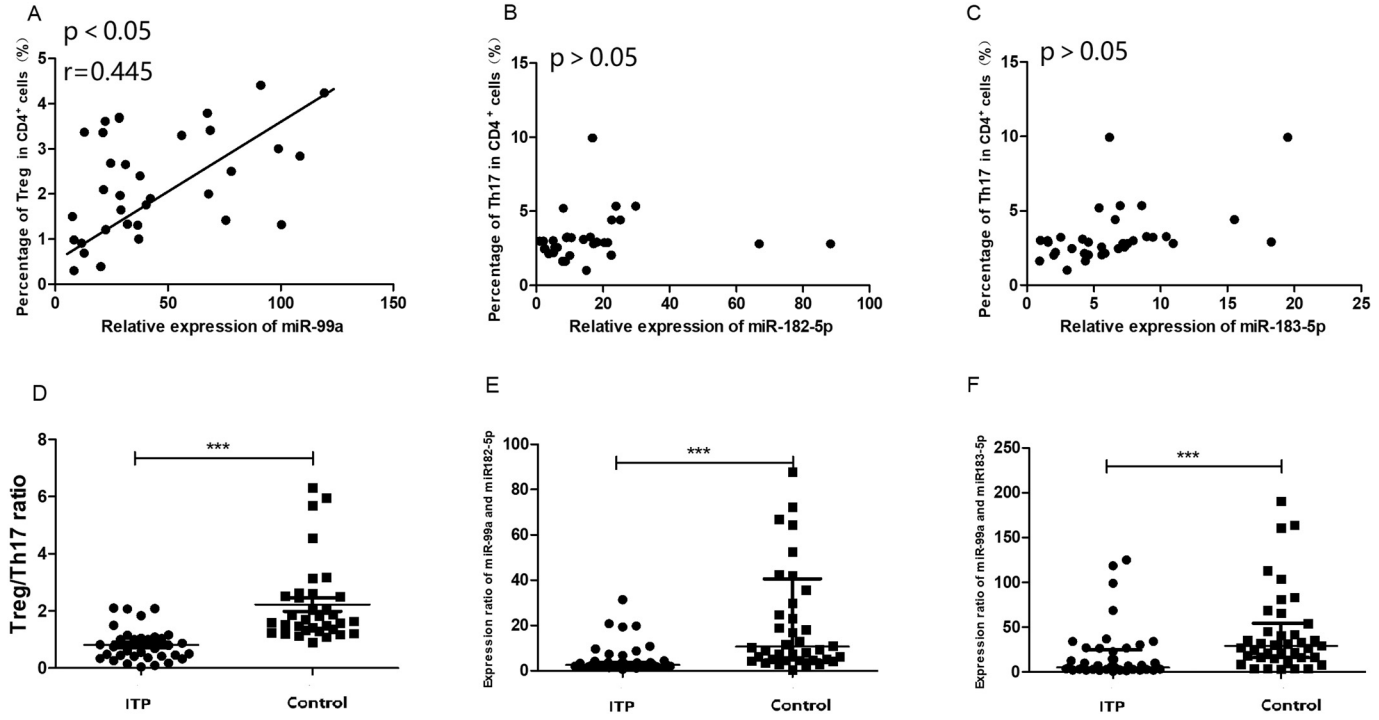

Fig. 3. Relationship between miRNAs and Th17/Treg subsets. (A–C) Relationship between Th17/Treg subsets and miR-99a, miR-182-5p, and miR-183-5p. (D) Ratio of Treg and Th17 cells in ITP patients and healthy controls. (E and F) Ratio of miR-99a/miR-182-5p or miR-99a/miR-183-5p in ITP patients and healthy controls.

3.3. Analysis between Th17/Treg related cytokines and miRNAs

To evaluate regulation of aberrant miRNAs on the function of $CD4^+$ cells, we measured IL-17A, a key cytokine of Th17, and IL-10 and TGF-β1, functional cytokines of Treg cells [30–33]. IL-17A expression was elevated in plasma from active ITP patients, but IL-10 and TGF-β1 expression decreased compared with controls (IL-17A, median 2.06 pg/ml vs 1.10 pg/ml, $p < 0.01$, Fig. 4B; IL-10, median 5.95 pg/ml vs 27.46 pg/ml, $p < 0.001$, Fig. 4A; TGF-β1, median 4.68 μg/ml vs 29.72 μg/ml, $p < 0.001$, Fig. 4C). We further demonstrated that IL-10 level correlated with miR-99a expression in patients with active ITP ($p < 0.01$, $r = 0.408$; Fig. 4D). However, miR-99a expression did not correlate significantly with TGF-β1 (Fig. 4E). In addition, there was no significant difference between IL-17 and miR-182-5p or miR-183-5p in ITP patients (Fig. 4F, G).

3.4. Regulation of Th17/Treg by miRNA

Intracellular cytokine staining showed that the frequency of Th17 was repressed by down-regulation of miR-183-5p, while up-regulated miR-99a expression with mimics promoted Treg in $CD4^+$ cells from ITP patients ($p < 0.01$, Fig. 5A, B; $p < 0.05$, Fig. 5C, D). However, miR-182-5p inhibitor did no influence Th17 differentiation. Warth et al. showed that miR-99a expression promoted Treg differentiation by repressing mTOR [34]. Besides, mTOR expression and its phosphorylation were reduced after miR-99a overexpression in $CD4^+$ T cells from ITP patients ($p < 0.05$; Fig. 5E–H).

3.5. miRNA expression and clinical characteristics

To determine whether miRNAs were associated with clinical characteristics, including platelet counts, severity, and corticosteroid sensitivity [24], we analyzed expression of miRNAs between different groups. miR-183-5p expression correlated negatively with platelet count ($p < 0.05$; $r = -0.348$; Fig. 6C). However, no significant correlation was found between platelet counts and miR-99a or miR-182-5p expression ($p > 0.05$; Fig. 6A, B).

When we studied severity of ITP, we found that miR-183-5p expression in CD4+ T cells from severe patients was significantly higher than in those from non-severe patients ($p < 0.05$; Fig. 6F). There was no significant difference in miR-99a and miR-182-5p between severe and non-severe ITP patients ($p > 0.05$; Fig. 6D, E). We also divided patients into corticosteroid-sensitive and corticosteroid-resistant groups according to the diagnosis guideline after corticosteroid treatment. We identified no significant difference in the expression of miRNAs between the sensitive and resistant groups ($p > 0.05$; Fig. 6G–I).

4. Discussion

Th17 and Treg cells are involved in the pathogenesis of ITP [35]. We found that the percentage of Th17 cells was increased while that of Treg cells was decreased in ITP patients. Many regulators are involved in the differentiation of Th17 and Treg cells, which serve as critical guardians of immune homeostasis. We hypothesized that miRNA was involved in the pathogenesis of ITP by regulating the differentiation of Th17 and Treg cells. We have shown that miR-99a was down-regulated, while miR-182-5p and miR-183-5p were up-regulated in the $CD4^+$ T cells from active ITP patients compared with healthy controls. Interestingly, we found the percentage of Treg cells correlated positively with miR-99a expression, which is accompanied by decreased percentage of Treg cells in ITP patients. In addition, Th17 and Treg cell differentiation improved after restoring miR-99a, miR-182-5p, and miR-183-5p expression. Other miRNAs, such as miR-96-5p, miR-17-5p, miR-146-5p, miR-155-5p, miR-181-5p, and miR-326, did not significantly affect regulation of Th17 and Treg cell differentiation.

A recent study suggested that miR-99a promoted Treg cells and inhibited Th17 differentiation by targeting mTOR in peripheral T cells, while miR-99a overexpression had no effect on thymic Treg development [34]. Consistent with this previous study, we found that miR-99a deficiency in $CD4^+$ lymphocytes impaired Treg cells while up-regulating Th17 cells from ITP patients. miR-99a expression correlated positively with Treg cell percentage in ITP patients. In a previous study,

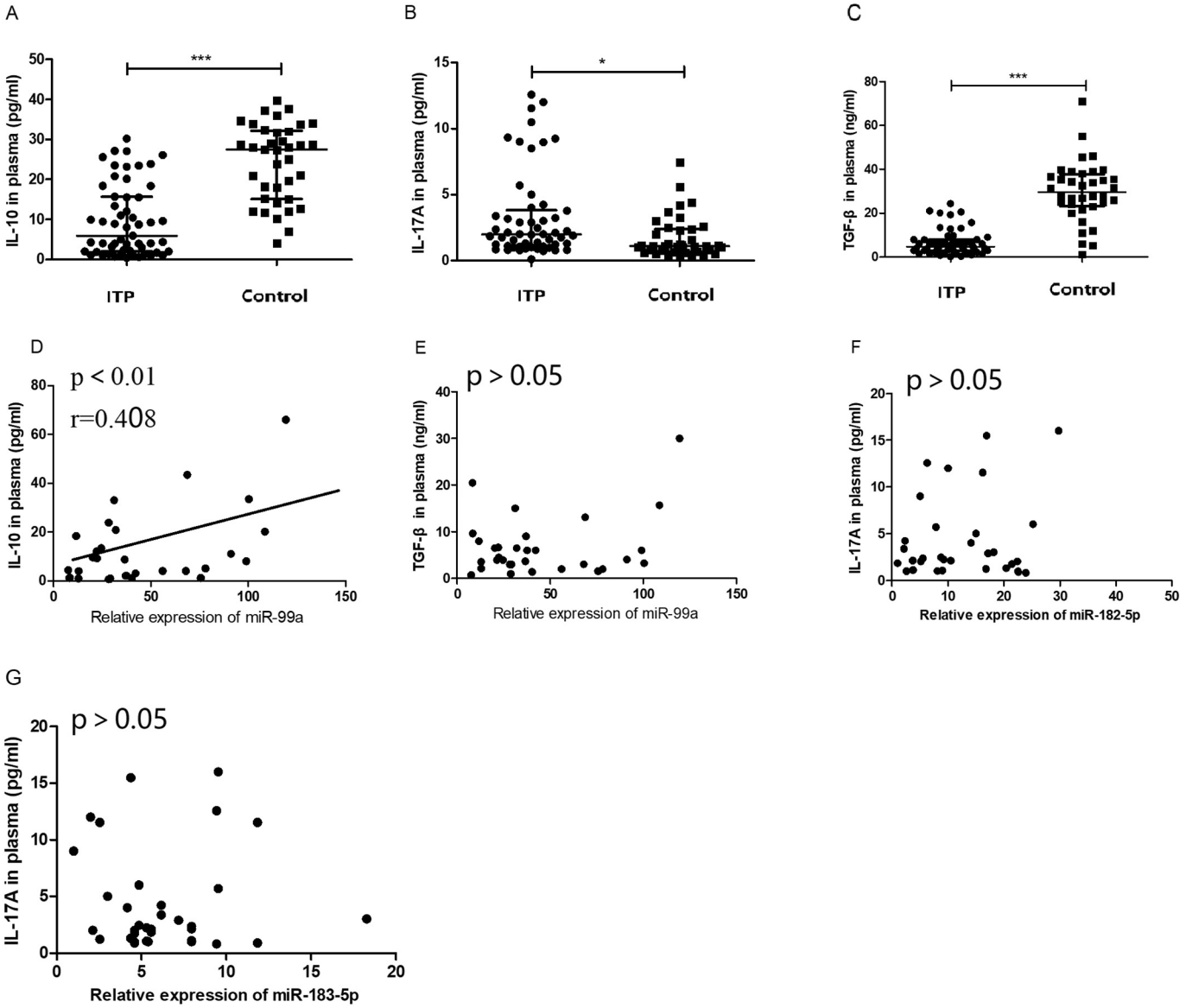

Fig. 4. Relationship between miRNAs and cytokines. (A–C) Expression of IL-10, IL-17A, and TGF-β1 in the plasma from ITP patients and healthy controls. (D and E) Linear correlation analysis between miR-99a and IL-10 or TGF-β1. (F and G) Relationship between IL-17A and miR-182-5p or miR-183-5p. Median with interquartile range.

miR-99a down-regulated human γδ T cell activation and cytotoxicity, suggesting that miR-99a plays an important role in T cell differentiation and function [36]. Furthermore, we found that IL-10 and TGF-β1 decreased while IL-17 increased in plasma of ITP patients, consistent with the aberrant Th17/Treg ratio. Moreover, miR-99a correlated positively with IL-10 expression, further demonstrating that miR-99a was involved in Treg cell differentiation. To clarify the function of miR-99a, we transferred miR-99a mimics into $CD4^+$ T cells from ITP patients to repair the level of miR-99a. The percentage of Treg cells increased after transfection. We concluded that miR-99a plays an important role in Treg cell differentiation.

To investigate the regulatory mechanism, we measured mTOR expression and found that mTOR expression decreased after transfection of miR-99a mimics in $CD4^+$ cells from ITP patients. Warth et al. found that miR-99a repressed mTOR expression cooperatively with miR-150 to promote Treg cell differentiation [34]. Damsky et al. showed that upregulating miR-99/100 contributed to the downregulation of mTOR and IGF1R signaling [37]. Many previous studies report results consistent with our findings.

In active ITP patients, miR-182-5p and miR-183-5p levels were elevated in $CD4^+$ T cells and the percentage of Th17 cells were increased. We hypothesized that miR-182-5p and miR-183-5p were critical regulators contributing to the development of Th17 cells. Ichiyama et al. found that the Dicer1-regulated miR-183 cluster containing miR-183-5p, miR-96-5p, and miR-182-5p, was highly expressed in Th17 cells and promoted pathogenic function of Th17 cells [38]. In addition, the miR-183 cluster modulated multiple sclerosis, another autoimmune disease, by inducing Th17 differentiation [39]. These previous studies support our hypothesis. To test our hypothesis, we silenced miR-182-5p and miR-183-5p in $CD4^+$ T cells from ITP patients and found that development of Th17 cells returned to normal. Though the previous studies reported that miR-96-5p promoted Th17 cell differentiation [38,39], miR-96-5p was inessential to the imbalance of Th17/Treg cells in our study.

miR-155 contributes to Th17 cell development by targeting Foxp3 [40]. miR-155 was highly elevated in mice with experimental autoimmune myocarditis; antagomir-155 treatment decreased Th17 immune response and reduced Th17-polarizing cytokines secreted by

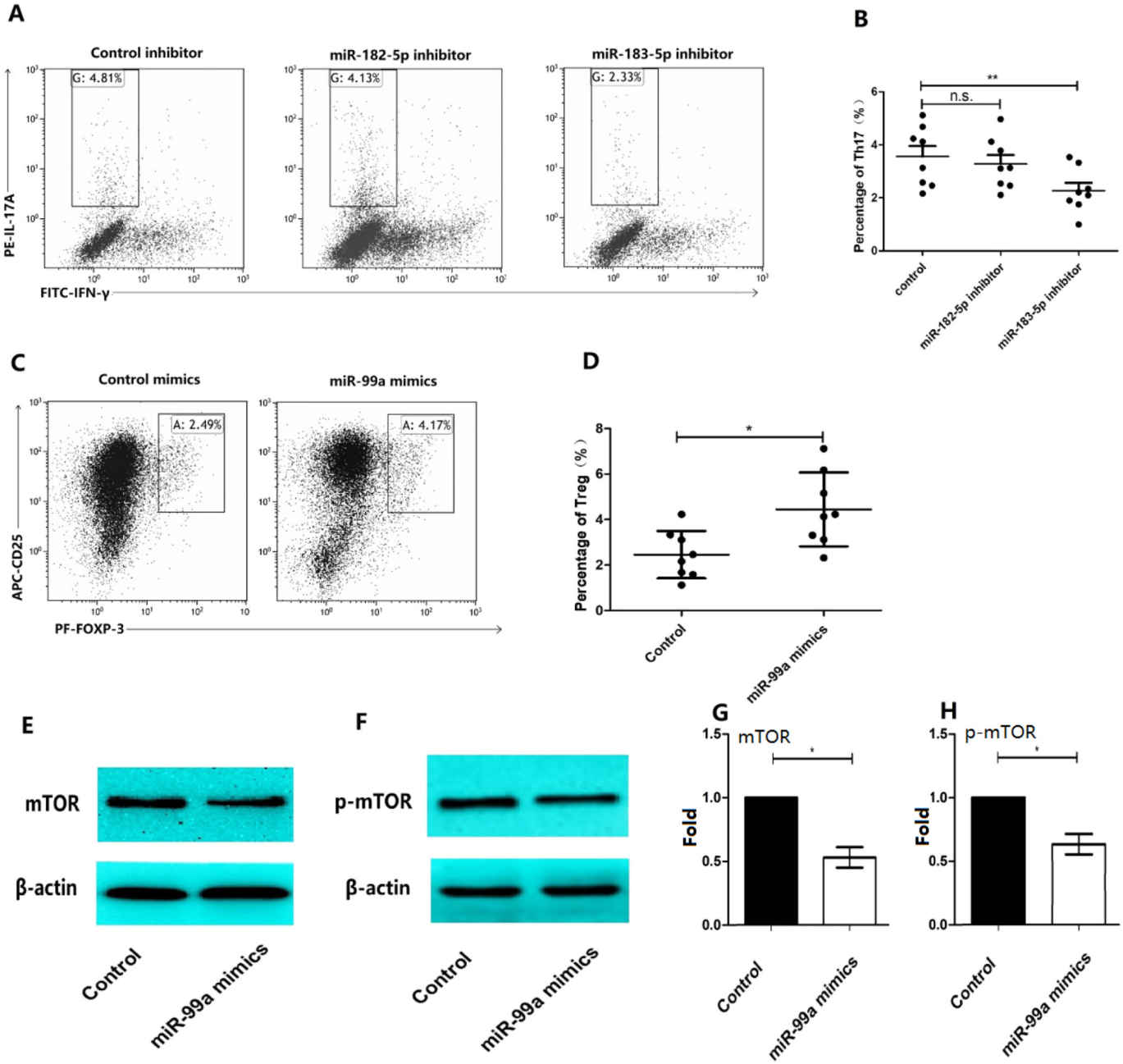

Fig. 5. Regulation of Treg or Th17 by miRNA. Isolated $CD4^+$ T cells from eight randomly selected ITP patients were transfected with miRNA mimics for miR-99a or miRNA inhibitor for miR-182-5p and miR-183-5p with negative control. After 4 days, the $CD4^+$cells were harvested and analyzed for Th17 and Treg cells differentiation by flow cytometry (A–D). (A) Gate "G": representative scattergrams of IL-17^+IFN-γ^- on $CD3^+$ $CD8^-$ T cells in control inhibitor, miR-182-5p inhibitor, or miR-183-5p inhibitor, respectively. (B) Percentage of Th17 cells in control group, miR-182-5p inhibitor group and miR-183-5p inhibitor group. (C) Gate "A": representative scattergrams of on $FOXP3^+$ $CD25^+$ on $CD4^+$ cells in control mimics or miR-99a mimics, respectively. (D) Percentage of Treg cells in control group and miR-99a mimics group. Median with interquartile range. (E–F) Representative images of immunoblotting for the expression of mTOR and p-mTOR in $CD4^+$ T cells. (G–H) Quantification of immunoblotting for the expression of mTOR and p-mTOR in $CD4^+$ T cells from 3 ITP patients. Results were normalized by β-actin and presented relative to those of negative control group, set as 1. Data show mean ± SEM.

dendritic cells [41]. As previously demonstrated, miR-155 affected the Th17 differentiation in other autoimmune diseases. To evaluate the function of miR-155 in the Th17/Treg imbalance in ITP pathogenesis, we tested the miR-155 in $CD4^+$ T cells from ITP patients and healthy controls. However, there was no significant difference in miR-155 expression between ITP and control groups. While previous studies found a close connection between the miRNAs and T cell subset imbalance [21,42,43], we found no significant association between Th17/Treg imbalance and miR-326, miR-181-5p, miR-17-5p, and miR-146-5p in ITP patients.

Importantly, our study demonstrated that miR-99a, miR-182-5p, and miR-183-5p were involved in the Th17/Treg imbalance, which contributed to the pathogenesis of ITP. miRNAs were also associated with clinical characteristics, such as corticosteroid sensitivity, platelet count and so on. miR-183-5p expression in severe ITP patients was significantly higher than that in non-severe patients. Besides, miR-183-5p expression correlated negatively with platelet count. miR-183-5p may be a valuable target for clinical application in ITP patients.

There were some limitations in our study. For example, we did not determine the specific mechanism that regulates the aberrant

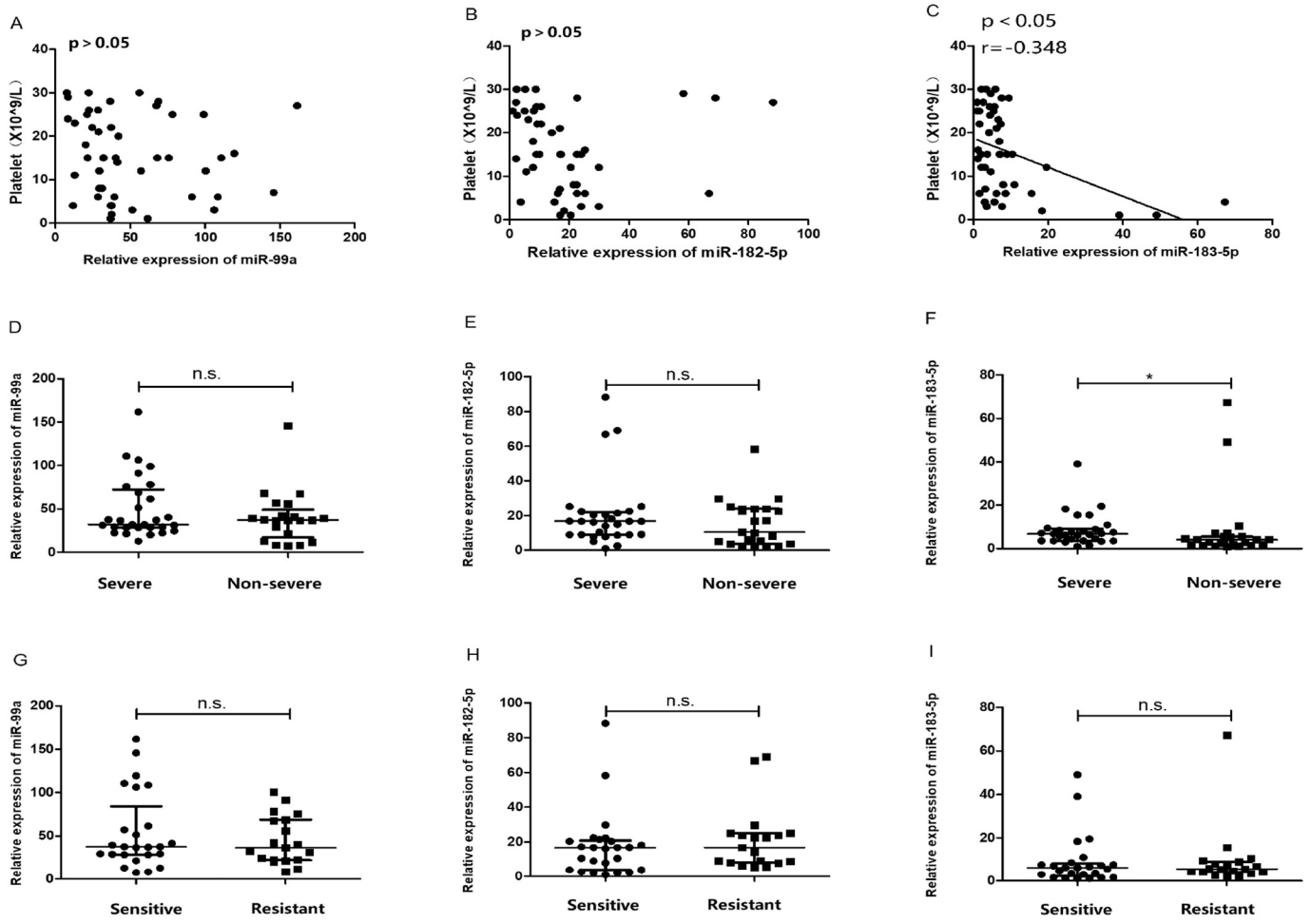

Fig. 6. Analysis between expression of miRNAs and clinical characteristics. (A–C) Relationship between platelet count and miR-99a, miR-182-5p, and miR-183-5p. (D–F) Expression of miR-99a, miR-182-5p, and miR-183-5p in severe and non-severe ITP patients. (G–I) Expression of miR-99a, miR-182-5p, and miR-183-5p in corticosteroid-sensitive and corticosteroid-resistant ITP patients. Median with interquartile range.

expression of miRNAs in ITP patients. Hoban et al. found that gut microbiome plays an important role in regulating miRNA profile in rodents [44]. Recently, several studies have identified that there is microbiome disturbance in ITP patients [45]. We would further explore whether there was relationship between the imbalance of microbiome and miRNAs in ITP patients. We also did not identify how miR-182-5p and miR-183-5p modulate differentiation of Th17 or contribute to the pathogenesis of ITP. We would like to do further studies about the functions of miRNAs in ITP.

In conclusion, we screened miRNAs in $CD4^+$ T cells and found aberrant expression of miR-99a, miR-182-5p, and miR-183-5p in ITP patients. miR-182-5p and miR-183-5p were involved in the differentiation of Th17 cells. miR-99a regulated the differentiation of Treg cells by targeting mTOR. miR-99a, miR-182-5p, and miR-183-5p were critically involved in the pathogenesis of ITP by regulating the balance of Th17/Treg.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2019.03.005.

Statement of prior presentation

Part of this study was presented as a poster with a title of "Aberrant Expression of microRNAs in CD4+ Cells May Contribute to the Imbalance of Th17/Treg Cells in Primary Immune Thrombocytopenia" (Abstract Code: 1140). This abstract won ASH Abstract Achievement Award at the 60th ASH Annual Meeting and Exposition organized by American Society of Hematology in San Diego, CA, December 1–4, 2018.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (grant numbers 81800112, 81770133, 81470284, 81770114, 81500094, and 81500095), Major Research plan of the National Natural Science Foundation of China (grant number 91442204), State Key Clinical Specialty of China for Blood Disorders and Tai Shan Scholar Foundation. Alexandra H. Marshall (Marshall Medical Communications) provided editorial assistance during manuscript preparation.

Authorship contributions

Mingqiang Hua conducted the study. Qi Feng helped to define the study objectives and to coordinate the study. Jun Peng, Ming Hou, and Linlin Shao performed the statistical analysis and interpreted the results, and Chunyan Wang and Ju Li wrote the first draft of the manuscript. All authors critically revised the manuscript and approved its final version.

Disclosure of conflict of interest

The authors state that they have no conflict of interest.

References

[1] F. Rodeghiero, et al., Standardization of terminology, definitions and outcome criteria in immune thrombocytopenic purpura of adults and children: report from an international working group, Blood 113 (11) (2009) 2386–2393.
[2] Z. Liu, et al., Pulsed high-dose dexamethasone modulates Th1-/Th2-chemokine imbalance in immune thrombocytopenia, J. Transl. Med. 14 (1) (2016) 301.
[3] Y. Hu, et al., Increased number of Tc17 and correlation with Th17 cells in patients with immune thrombocytopenia, PLoS One 6 (10) (2011) e26522.
[4] A.M. Rocha, et al., The levels of IL-17A and of the cytokines involved in Th17 cell commitment are increased in patients with chronic immune thrombocytopenia, Haematologica 96 (10) (2011) 1560–1564.
[5] T. Nishimoto, M. Kuwana, CD4+CD25+Foxp3+ regulatory T cells in the pathophysiology of immune thrombocytopenia, Semin. Hematol. 50 (Suppl. 1) (2013) S43–S49.
[6] B. Liu, et al., Abnormality of CD4(+)CD25(+) regulatory T cells in idiopathic thrombocytopenic purpura, Eur. J. Haematol. 78 (2) (2007) 139–143.
[7] E. Bettelli, et al., Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells, Nature 441 (7090) (2006) 235–238.
[8] M. Noack, P. Miossec, Th17 and regulatory T cell balance in autoimmune and inflammatory diseases, Autoimmun. Rev. 13 (6) (2014) 668–677.
[9] R.M. O'Connell, et al., Physiological and pathological roles for microRNAs in the immune system, Nat. Rev. Immunol. 10 (2) (2010) 111–122.
[10] J. Hou, et al., MicroRNA-146a feedback inhibits RIG-I-dependent type I IFN production in macrophages by targeting TRAF6, IRAK1, and IRAK2, J. Immunol. 183 (3) (2009) 2150–2158.
[11] K. Ichiyama, et al., The microRNA-183-96-182 cluster promotes T helper 17 cell pathogenicity by negatively regulating transcription factor Foxo1 expression, Immunity 44 (6) (2016) 1284–1298.
[12] M. Kastle, et al., microRNA cluster 106a~363 is involved in T helper 17 cell differentiation, Immunology 152 (3) (2017) 402–413.
[13] H.Y. Yang, et al., MicroRNA-17 modulates regulatory T cell function by targeting co-regulators of the Foxp3 transcription factor, Immunity 45 (1) (2016) 83–93.
[14] S. Jiang, et al., Molecular dissection of the miR-17-92 cluster's critical dual roles in promoting Th1 responses and preventing inducible Treg differentiation, Blood 118 (20) (2011) 5487–5497.
[15] S.C. Warth, et al., Induced miR-99a expression represses Mtor cooperatively with miR-150 to promote regulatory T-cell differentiation, EMBO J. 34 (9) (2015) 1195–1213.
[16] Y. Chung, et al., Critical regulation of early Th17 cell differentiation by interleukin-1 signaling, Immunity 30 (4) (2009) 576–587.
[17] M. Xu, et al., MiR-155 contributes to Th17 cells differentiation in dextran sulfate sodium (DSS)-induced colitis mice via Jarid2, Biochem. Biophys. Res. Commun. 488 (1) (2017) 6–14.
[18] R.M. O'Connell, et al., MicroRNA-155 promotes autoimmune inflammation by enhancing inflammatory T cell development, Immunity 33 (4) (2010) 607–619.
[19] J. Heyn, et al., miR-124a and miR-155 enhance differentiation of regulatory T cells in patients with neuropathic pain, J. Neuroinflammation 13 (1) (2016) 248.
[20] S. Ghorbani, et al., MicroRNA-181 variants regulate T cell phenotype in the context of autoimmune neuroinflammation, Front. Immunol. 8 (2017) 758.
[21] C. Du, et al., MicroRNA miR-326 regulates TH-17 differentiation and is associated with the pathogenesis of multiple sclerosis, Nat. Immunol. 10 (12) (2009) 1252–1259.
[22] C. Chen, et al., Dysregulated microRNA involvement in multiple sclerosis by induction of T helper 17 cell differentiation, Front. Immunol. 9 (2018) 1256.
[23] D. Provan, et al., International consensus report on the investigation and management of primary immune thrombocytopenia, Blood 115 (2) (2010) 168–186.
[24] Consensus of Chinese experts on diagnosis and treatment of adult primary immune thrombocytopenia (version 2016), Zhonghua Xue Ye Xue Za Zhi 37 (2) (2016) 89–93.
[25] L. Liu, et al., The aberrant expression of microRNAs and correlations with T cell subsets in patients with immune thrombocytopenia, Oncotarget 7 (47) (2016) 76453–76463.
[26] M. Jernås, et al., MicroRNA regulate immunological pathways in T-cells in immune thrombocytopenia (ITP), Blood 121 (11) (2013) 2095–2098.
[27] J.-Q. Li, et al., MicroRNA-125-5p targeted CXCL13: a potential biomarker associated with immune thrombocytopenia, Am. J. Transl. Res. 7 (4) (2015) 772–780.
[28] S. Yu, et al., Inactivation of Notch signaling reverses the Th17/Treg imbalance in cells from patients with immune thrombocytopenia, Lab. Investig. 95 (2) (2015) 157–167.
[29] H. Zhong, et al., CD16+ monocytes control T-cell subset development in immune thrombocytopenia, Blood 120 (16) (2012) 3326–3335.
[30] P. Hsu, et al., IL-10 potentiates differentiation of human induced regulatory T cells via STAT3 and Foxo1, J. Immunol. 195 (8) (2015) 3665–3674.
[31] M.G. Roncarolo, et al., Interleukin-10-secreting type 1 regulatory T cells in rodents and humans, Immunol. Rev. 212 (2006) 28–50.
[32] B. Singh, et al., Modulation of autoimmune diseases by interleukin (IL)-17 producing regulatory T helper (Th17) cells, Indian J. Med. Res. 138 (5) (2013) 591–594.
[33] A. Carambia, et al., TGF-beta-dependent induction of CD4(+)CD25(+)Foxp3(+) Tregs by liver sinusoidal endothelial cells, J. Hepatol. 61 (3) (2014) 594–599.
[34] S.C. Warth, et al., Induced miR-99a expression represses Mtor cooperatively with miR-150 to promote regulatory T-cell differentiation, EMBO J. 34 (9) (2015) 1195–1213.
[35] L. Ji, et al., The ratio of Treg/Th17 cells correlates with the disease activity of primary immune thrombocytopenia, PLoS One 7 (12) (2012) e50909.
[36] Y. Zhu, et al., miR-125b-5p and miR-99a-5p downregulate human gammadelta T-cell activation and cytotoxicity, Cell. Mol. Immunol.16(2), 2018, 112-125. 16 (2) (2018) 112–125.
[37] W. Damsky, et al., mTORC1 activation blocks BrafV600E-induced growth arrest but is insufficient for melanoma formation, Cancer cell 27 (1) (2015) 41–56.
[38] K. Ichiyama, et al., The microRNA-183-96-182 cluster promotes T helper 17 cell pathogenicity by negatively regulating transcription factor Foxo1 expression, Immunity 44 (6) (2016) 1284–1298.
[39] C. Chen, et al., Dysregulated microRNA involvement in multiple sclerosis by induction of T helper 17 cell differentiation, Front. Immunol. 9 (2018) 1256.
[40] S. Kohlhaas, et al., Cutting edge: the Foxp3 target miR-155 contributes to the development of regulatory T cells, J. Immunol. 182 (5) (2009) 2578–2582.
[41] L. Yan, et al., Inhibition of microRNA-155 ameliorates experimental autoimmune myocarditis by modulating Th17/Treg immune response, J. Mol. Med. (Berl) 94 (9) (2016) 1063–1079.
[42] H.-Y. Yang, et al., MicroRNA-17 modulates regulatory T cell function by targeting co-regulators of the Foxp3 transcription factor, Immunity 45 (1) (2016) 83–93.
[43] S. Jiang, et al., Molecular dissection of the miR-17-92 cluster's critical dual roles in promoting Th1 responses and preventing inducible Treg differentiation, Blood 118 (20) (2011) 5487–5497.
[44] A.E. Hoban, et al., Microbial regulation of microRNA expression in the amygdala and prefrontal cortex, Microbiome 5 (1) (2017) 102.
[45] G.H. Frydman, et al., Helicobacter pylori eradication in patients with immune thrombocytopenic purpura: a review and the role of biogeography, Helicobacter 20 (4) (2015) 239–251.